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Acceleration of Reactivation of Reduced Bovine Pancreatic Ribonuclease by a Microsomal System from Rat Liver

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Recent studies of protein biosynthesis have indicated that the ribosome is the site at which amino acids are assembled into polypeptide chains (1). On the basis of experiments with rabbit reticulocytes and hen oviduct tissue, the rate of polypeptide chain growth appears to be approximately 1 residue per second (2, 3). Although much information is now available concerning the mechanism of this initial phase of protein biosynthesis, little is known about the conversion of the polypeptide chains to the characteristic and highly complex three-dimensional structures of the corresponding protein molecules, a step that is thought to occur rapidly. Indeed, even the subcellular environment in which this conversion is accomplished is not known. Previous work on the reoxidation of bovine pancreatic ribonuclease (RNase) (4, 5) after reduction of disulfide bridges and disruption of tertiary structure has shown that the resumption of native configuration occurs spontaneously under appropriate conditions. It has been concluded, therefore, that no special genetic information, beyond that contained in the amino acid sequence, is required for the proper folding of the molecule and for the formation of the "correct" disulfide bonds.

In a recent study (6), the kinetics of reactivation of RNase during air reoxidation of the reduced form of the enzyme were examined. By choosing the lowest protein concentration technically feasible (0.01 mg per ml), a relatively high pH (8.2), and the optimal temperature (24°), it was possible to reduce the half-time for the reactivation of RNase to approximately 20 minutes. The presence of such compounds as cysteine, β-mercaptoethanol, RNA, bovine serum albumin, native RNase, and orthophosphate ions in the reoxidation medium did not further reduce this time. In order to determine whether there are systems *in vivo* capable of accelerating the reoxidation process, the effects of a rat liver homogenate were studied. This report presents the results of that study, and outlines the general characteristics of a subcellular system that greatly accelerates the reactivation of reduced RNase. Fractionation of the rat liver homogenate revealed that this system consists of both microsomes and a soluble nonprotein factor or factors.

EXPERIMENTAL PROCEDURE

Analytical Techniques—A Beckman DU spectrophotometer was used for determination of the absorption at 280 mμ of column effluents and for the routine assay of RNase (7). RNase assays by the method of Kunitz (8) were performed with the use of a Cary model 14 recording spectrophotometer. Protein concentrations of particle suspensions were determined by the technique of Lowry *et al.* (9).[1] Final adjustments of the pH of all reoxidation mixtures were monitored with a Radiometer PHM 22p pH meter.

Fractionation of Rat Liver—Twenty male albino Sprague-Dawley rats, each weighing 120 to 150 g, were used for each preparation. The rats were anesthetized with ether and killed by exsanguination. The livers were removed and immediately immersed in an ice-cold solution of Tris-chloride (0.01 M) (reagent grade, Sigma Chemical Company) and sucrose (0.25 M) (reagent grade, Baker and Adamson Company), pH 7.8. This Tris-sucrose solution was used throughout the fractionation procedure. All manipulations were carried out at 3°. Each liver was minced with a pair of scissors and homogenized for approximately 10 seconds with a Potter-Elvehjem glass-Teflon homogenizer. Differential centrifugation was carried out by a modification of the method of Siekevitz (10), as summarized schematically in Fig. 1. The combined homogenates, in a final volume of 600 ml, were centrifuged at 1,500 r.p.m. in a refrigerated International centrifuge (No. 269 head) for 15 minutes to remove whole cells, cell walls, and connective tissue. The supernatant fluid was centrifuged at 17,000 × *g* for 20 minutes in a Spinco model L ultracentrifuge with use of a No. 30 rotor. The mitochondria (R-I fraction) obtained as the residue were washed once and resuspended in Tris-sucrose solution at a final protein concentration of 30 mg per ml. The supernatant fluid (S-I fraction) was centrifuged at 105,000 × *g* for 2 hours, and the supernatant fluid (S-II fraction), with a protein concentration of 10.5 mg per ml, was decanted and stored at −20°. The residue from the latter centrifugation, consisting of microsomes, was washed once by homogenization in Tris-sucrose solution and centrifugation at 105,000 × *g* for 2 hours. The translucent pellet of microsomes thus obtained (R-II fraction) was homogenized in Tris-sucrose solution at a final protein concentration of 16.5 mg per ml and stored at −20°.

Microsomes (R-II fraction) and microsomal supernatant fluid (S-II fraction) were also prepared in 0.88 M sucrose by the method of Siekevitz (10).

Preparation of Rat Liver Ribosomes—Ribosomes were prepared from 100 mg of the R-II fraction by the method of Siekevitz (11), with reagent grade sodium deoxycholate (Baltimore Chemical Company). The ribosomes were homogenized in 1.0 ml of Tris-sucrose solution and used at once.

Fractionation of S-II Fraction—Fifty milliliters of the S-II

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[1] Protein determinations were kindly performed by Mr. Anthony Morrissey.

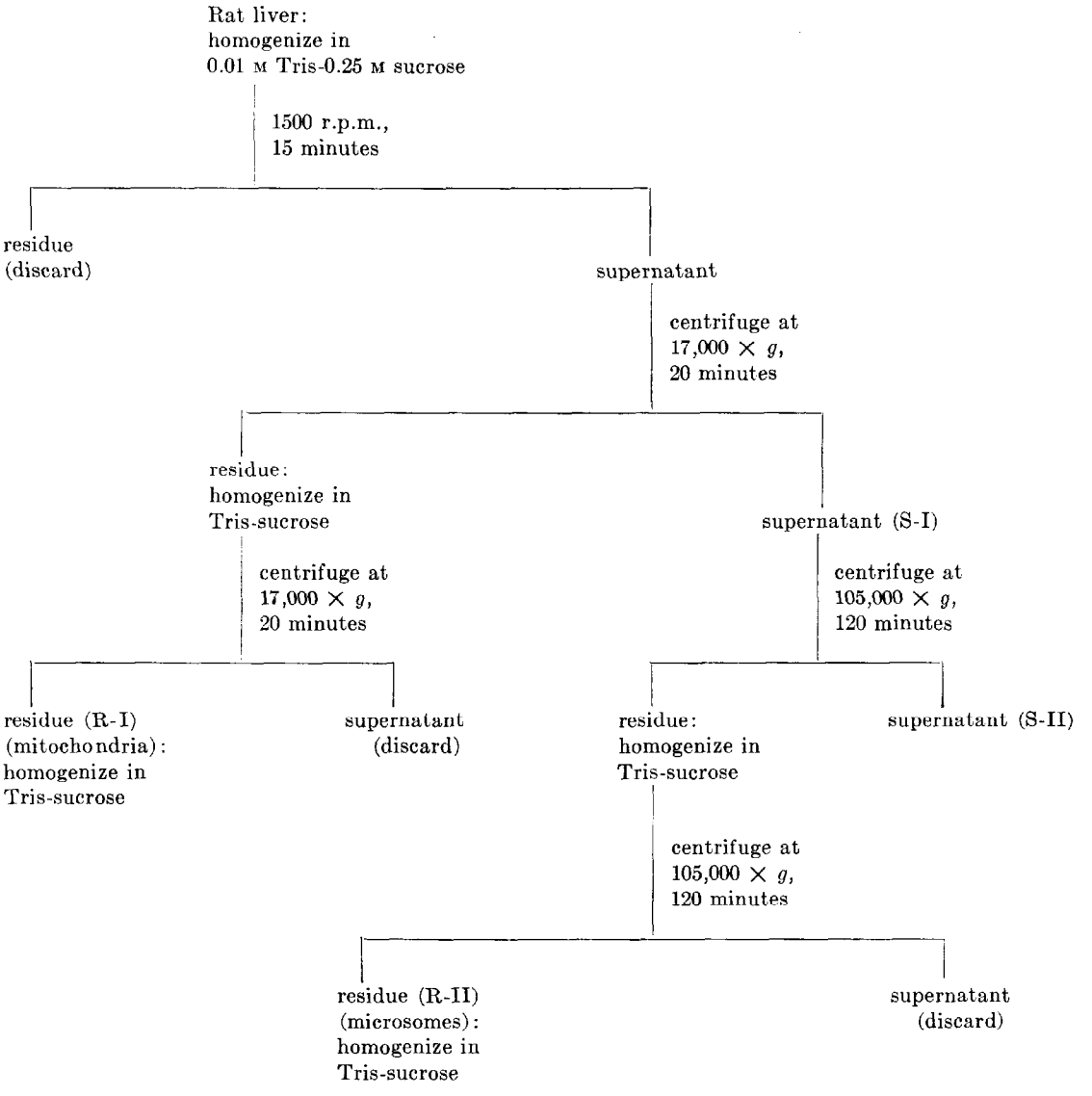

FIG. 1. Flow sheet for the fractionation of rat liver at 3°

fraction were lyophilized and redissolved in 5 ml of water. This concentrated solution was subjected to gel filtration on a column of Sephadex G-25, 2.5 × 50 cm (Pharmacia, Uppsala, Sweden), previously equilibrated with 0.01 M Tris-chloride buffer, pH 7.8. A graph showing the results of this procedure is presented in Fig. 2. The material represented by Peak A-1 formed a deep red band on the Sephadex and appeared in the effluent "front." The material represented by Peak B-1 appeared in the effluent at the known "salt volume" for this column. Peak C-1 represents material that was retarded on the Sephadex and emerged after the salt peak. The fractions corresponding to Peaks A-1, B-1, and C-1 were pooled separately, lyophilized, and redissolved in 5 ml of water. Each of the three solutions was then subjected once again to gel filtration. The results obtained are shown graphically in Fig. 3. The three fractions, which had been poorly separated on the first passage through Sephadex, were well resolved on the second. The effluents corresponding to the main peak (A-2, B-2, or C-2) in each of the three cases were pooled, lyophilized, and redissolved in 15 ml of water for assay.

Another fractionation procedure applied to the S-II fraction combined isoelectric precipitation with acid and heat denaturation of proteins. A solution of the S-II fraction at 24° was adjusted to pH 4.5 by the addition of 2 N HCl, and the heavy orange precipitate that formed was removed by centrifugation. The pH of the supernatant fluid was adjusted to 2.0 by the addition of more HCl, but no further precipitation occurred. The pH was adjusted, by the addition of 2 N KOH, to 5.2 and then to 7.8, and the precipitate that formed at each step was removed by centrifugation. The supernatant fluid was then heated in a boiling water bath for 3 minutes and allowed to cool slowly to 24°. The small amount of flocculent precipitate that formed during the heating was removed by centrifugation, and the final supernatant fluid, with a protein concentration of 0.25 mg per ml, was stored at −20°. By this procedure, 97.5% of the protein was removed from the S-II fraction.

Reduction of Ribonuclease—Five times recrystallized bovine pancreatic RNase, type III (Lot RB12-086), was purchased from the Sigma Chemical Company. Chromatography on carboxymethyl cellulose (CM-cellulose) by the method of Åqvist

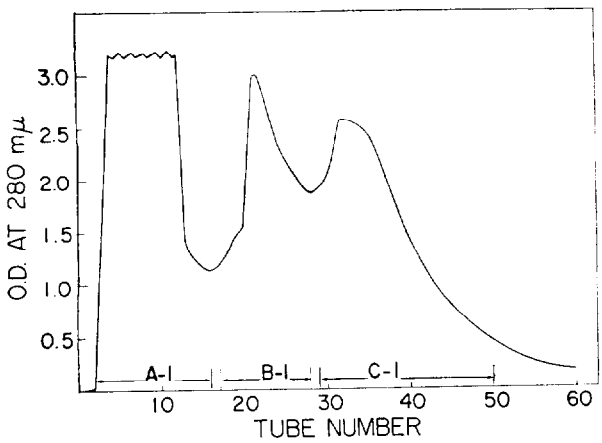

FIG. 2. Elution pattern from gel filtration of the S-II fraction. Fifty milliliters of the S-II fraction were concentrated by lyophilization, redissolved in 5 ml of water, and applied to a column of Sephadex G-25, 2.5 × 50 cm, previously equilibrated with 0.01 M Tris-chloride buffer, pH 7.8. Effluent fluids corresponding to the three peaks (A-1, B-1, and C-1), were pooled as shown at the *bottom* of the graph.

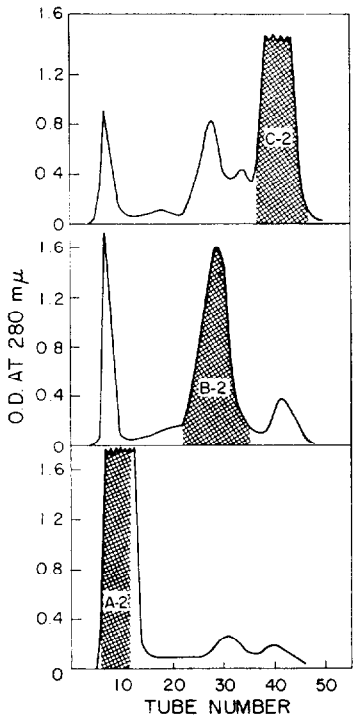

FIG. 3. Elution patterns from gel filtration of Peaks A-1, B-1, and C-1. Pooled effluents corresponding to the three peaks shown in Fig. 2 were lyophilized and redissolved in 5 ml of water. Each solution was then subjected to gel filtration on a column of Sephadex G-25, 2.5 × 50 cm, previously equilibrated with 0.01 M Tris-chloride buffer, pH 7.8. Effluents corresponding to the *shaded area* in each of the three peaks (A-2, B-2, and C-2) were pooled, lyophilized, and redissolved in 15 ml of water to be tested for their ability to replace the S-II fraction in reoxidation mixtures containing microsomes.

and Anfinsen (12) revealed that this lot contained no significant amount of nucleotides and consisted predominantly of "A" with a minor amount of the active "B" peak. The methods for complete reduction of the enzyme by treatment with β-mercaptoethanol (Eastman, white label) in 8 M urea, and for the separation of the reduced protein from these reagents by gel filtration, have been described previously (4).

Reoxidation of Ribonuclease—Unless otherwise specified, reoxidation of RNase was carried out as follows. The reduced enzyme, at a concentration of 0.018 mg per ml, was reoxidized in a final volume of 5.5 ml in a 50-ml Erlenmeyer flask, at 37° and pH 7.4 ± 0.05, in 0.05 M Tris chloride buffer. When liver fractions were included in the reoxidation mixture, the following quantities were used (except as otherwise specified): mitochondria (R-I fraction), microsomes (R-II fraction), or ribosomes, 0.5 ml; S-II fraction, 2.25 ml; fractions derived from the S-II fraction by gel filtration, 2.0 ml; supernatant obtained from the S-II fraction after isoelectric precipitation and acid and heat denaturation of proteins, 2.25 ml. The "standard reoxidation mixture" contained microsomes, 0.5 ml; S-II fraction, 2.25 ml; and 0.1 M Tris-chloride buffer, 2.25 ml.

Assay for Enzymic Activity—Enzymic activity was assayed by digestion of yeast RNA (13) at pH 5.0, followed by precipitation with uranyl acetate-perchloric acid solution (7). Duplicate aliquots were assayed at various times during the reoxidation process. Assays utilizing the method of Kunitz (8) were performed in a few instances, as noted below. These assays were carried out at pH 8.0.

RESULTS

Reactivation of Reduced RNase in Presence of Rat Liver Fractions—After initial studies showed that the rate of reactivation of reduced RNase was greatly accelerated by a crude rat liver homogenate, various rat liver fractions were investigated separately and in combination. The results of these experiments are summarized in Table I. Mitochondria (R-I fraction) were totally without effect on the kinetics of reactivation, whereas the supernatant fluid (S-I fraction) retained the full effect exerted

TABLE I

Reactivation of reduced RNase in presence of rat liver fractions

Reduced RNase was reoxidized at a concentration of 0.018 mg per ml in 0.05 M Tris-chloride buffer at pH 7.4 and 37°. Also included in the reoxidation mixtures were the following quantities of rat liver homogenate or fractions thereof: in Experiment 2, 4.5 ml of rat liver homogenate; in Experiments 3 and 8, 0.5 ml of the mitochondrial suspension (R-I); in Experiment 4, 1.0 ml of the S-I fraction; in Experiments 5 and 7, 0.5 ml of the microsomal suspension (R-II); and in Experiments 6, 7, and 8, 2.25 ml of the S-II fraction. The final volume in all cases was 5.5 ml. Aliquots were removed from the reoxidation mixtures and assayed by digestion of yeast RNA immediately after addition of reduced RNase and again 20 minutes later. The results given in the table are the differences in the optical density values obtained in the assays performed at zero time and at 20 minutes.

Experiment	Rat liver fraction	ΔO.D. at 260 mμ in 20 minutes
1	None	0.011
2	Liver homogenate	0.092
3	R-I (mitochondria)	0.009
4	S-I	0.118
5	R-II (microsomes)	0.011
6	S-II	0.001
7	S-II + R-II	0.176
8	S-II + R-I	0.002

by the crude homogenate. Neither washed microsomes (R-II fraction) nor the supernatant fluid (S-II fraction), the two fractions derived from the S-I fraction, were able to accelerate the reactivation. Upon recombination of these two fractions, however, the full effect was restored. It thus became clear that the liver system responsible for accelerating the conversion of reduced RNase to the active form of the enzyme consists of both microsomes and one or more factors in the S-II fraction. As shown in Table I, no combination of liver fractions other than that of microsomes plus the S-II fraction was active. The data in Table I are shown only to demonstrate the presence or absence of stimulatory effect in the various rat liver fractions. The degree of stimulation, on the other hand, can be appreciated only by examination of the kinetics of the reactivation process (see below under "Effect of Variations in Concentrations of Rat Liver Fractions" and Fig. 5).

In order to determine the nature of the active component or components in the S-II fraction, several fractionation procedures were employed. Isoelectric precipitation and acid and heat denaturation of proteins in the S-II fraction were carried out as described above. The final supernatant fluid, which contained only 2.5% of the protein originally present, retained the full effect of the S-II fraction.

Fractionation of the S-II fraction by gel filtration, accomplished as described above, gave the elution patterns shown in Figs. 2 and 3. The material of each of the main peaks was tested to determine whether it could replace the S-II fraction in reoxidation mixtures containing microsomes. The results of these experiments are shown in Table II. The components contained in both Peak A-2 (the "protein" peak) and Peak B-2 (the "salt volume" peak) were active, whereas those in Peak C-2 were not. By subjecting the material of Peak B-2 to gel filtration a third time, it was shown that the activity of this fraction was confined to the material of the main peak. The position at which this material emerged from Sephadex indicates a molecular weight no larger than 1000 to 2000.

A dialysis experiment was performed to estimate further the size of the active component(s) of the S-II fraction. The S-II fraction and solutions of Peaks A-2 and B-2 and of the supernatant fluid obtained after acid treatment of Peak A-1 were dialyzed separately against 0.01 M Tris-chloride buffer, pH 7.8, for 12 hours at 3°. They were then tested for their ability to replace the S-II fraction in reoxidation mixture containing microsomes. The results are shown in Table II. No appreciable activity was lost from Peak A-2, a moderate amount was lost from the acid-treated A-1 material, virtually all activity was lost from Peak B-2, and approximately 50% was lost from the S-II fraction. It seems likely, therefore, that the active component(s) in the S-II fraction is a small, freely dialyzable molecule, a portion of which is probably bound to protein. Treatment with acid seems to cause partial dissociation of the active component(s) from the protein.

Supernatant fluid prepared from beef heart homogenates (14)[2] was found to replace completely the S-II fraction in reoxidation mixtures containing rat liver microsomes. It was not active in the absence of microsomes.

Assay Controls—During the course of the present study, it was noted that standard solutions of native RNase had higher activities when assayed (by both assay techniques) after incubation with liver fractions than when assayed after incubation in the Tris buffer. In order to estimate the true degree of conversion of reduced RNase to active enzyme during reoxidation experiments, a calibration curve was constructed in the following manner. Solutions of native RNase of various known concentrations were assayed before and after incubation with each of the liver fractions. A curve was constructed in which the activity after incubation was plotted as a function of the activity before incubation, *i.e.* the activity in Tris buffer. It was found that incubation with all fractions shown in Fig. 1, as well as the acid- and heat-treated S-II fraction, separately and in combination, produced about the same degree of elevation of activity, amounting to approximately 30 to 50%. The phenomenon was independent of the length of the incubation period, and could also be produced by variations in the composition of the buffer in the reoxidation mixtures.[3] In any case, it does not enter into

[2] Kindly supplied by Dr. Archie L. Smith, Institute for Enzyme Research, Madison, Wisconsin.

TABLE II

Effect of fractionation of S-II fraction

Reduced RNase, at a concentration of 0.018 mg per ml, was reoxidized in a total volume of 5.5 ml at pH 7.4 and 37°. In all cases the reoxidation mixture contained Tris-chloride buffer at a final concentration of 0.05 M and 0.5 ml of the microsomal suspension. Also included were 2.25 ml of the S-II fraction or 2.0 ml of the various components thereof, as indicated in the table. Assays for enzymic activity with yeast RNA substrate were performed immediately after the addition of reduced RNase, and again 20 minutes later. The extent of reactivation achieved in 20 minutes is given as a percentage of that achieved when the S-II fraction (before dialysis) was used.

Component of S-II fraction	Relative activity	
	Before dialysis	After dialysis
	%	%
S-II	100	51
Peak A-1	40	
Peak A-2	59	49
Peak B-1	73	
Peak B-2	62	8
Peak C-1	7	
Peak C-2	13	
Peak A-1 (acid-treated)*	60	28

* The material of Peak A-1 was treated in the same manner as described for the isoelectric precipitation and acid and heat denaturation of protein in the S-II fraction (see "Fractionation of S-II Fraction"). The final supernatant fluid was then tested for its ability to replace the S-II fraction in the reoxidation mixture containing microsomes.

[3] Similar increases in activity over the value obtained with 0.05 M Tris buffer were noted after prior incubation of native RNase in 1% bovine serum albumin, 0.15 M calcium chloride, 0.2 M ammonium bicarbonate, 0.1 M sodium oxalate, or 0.1 M sodium phosphate buffer (pH 7.6). Initial incubation in water, 0.02 M sodium phosphate buffer, 0.1 M sodium acetate buffer (pH 5.0), or 0.07 M barbital buffer (pH 8.6) gave results equal to those obtained in Tris buffer, *i.e.* did not increase the activity. Prior incubation in potassium chloride at various concentrations ranging from 0.1 to 1.0 M demonstrated a progressive increase in activity, which amounted to approximately 50% at the highest concentration. On the basis of these observations, it appears likely that proteins, or salts at high ionic strengths, prevent loss of RNase from dilute solutions during the period before assay. This loss may be due to adsorption of the enzyme to the surface of the glass vessel (*cf.* (15)).

TABLE III

Effect of various substances on complete system

Reduced RNase was reoxidized at a concentration of 0.018 mg per ml in the "standard reoxidation mixture" (containing microsomes and the S-II fraction). Additions of TPNH, GSH, and GSSH were made as shown in the table. The final concentration of these components was 10^{-3} M. Aliquots were removed from the reoxidation mixtures and assayed immediately after addition of the reduced enzyme, and again 20 minutes later. The differences between the zero time activities and the 20-minute activities are expressed relative to that observed when no additions were made to the "standard reoxidation mixture."

Additions to "standard reoxidation mixture"	Relative activity
	%
None	100
TPNH, 10^{-3} M	103
GSH, 10^{-3} M	97
GSSG, 10^{-3} M	60
GSSG, 10^{-3} M + TPNH, 10^{-3} M	103

an interpretation of the relative effects of the various fractions on the reactivation process, since it concerns only the absolute *quantity* of activity, and not the *kinetics* of reactivation. Throughout this report, all values for enzyme activities observed in the presence of liver fractions have been converted, according to the calibration curve mentioned above, to give the equivalent activities in Tris buffer. Highly purified RNase A, obtained from RNase Lot 381-059 (Armour and Company) by chromatography and rechromatography on CM-cellulose (12),[4] did not differ from the RNase Lot RB12-086 (Sigma Chemical Company) used throughout the present study either with respect to the phenomenon of increased activity after incubation with liver fractions or with respect to the kinetics of reactivation after complete reduction of its disulfide bonds.

A series of control experiments was performed to test the possibility that the increase in enzyme activity measured during the reactivation of reduced RNase was due to some other endogenous activity, such as the latent RNase of rat liver microsomes (16). When a known quantity of native RNase was added to the "standard reoxidation mixture" in the absence of any reduced enzyme, aliquots removed over a 40-minute period did, on a few occasions, show an increase in activity with time. The increase, when present, amounted to not more than 5% of that observed for an equivalent amount of the reduced enzyme in an equal time period. No "RNase" activity was obtained when either reduced polyalaninated lysozyme[5] or performic acid-oxidized RNase, each at a final concentration of 0.018 mg per ml, was added to a "standard reoxidation mixture" in the absence of reduced RNase. Thus, even the small amount of latent activity occasionally elicited by native RNase is not elicited by an inactive molecule with essentially the same amino acid sequence or by an unrelated protein chain with the same number of free sulfhydryl groups as reduced RNase. The subtraction of the zero time reading, *i.e.* the activity present immediately after the addition of reduced enzyme to the microsomal system, from all subsequent readings eliminates from consideration any RNase activity endogenous to the liver preparations. Furthermore, once an aliquot from the reactivation mixture has been removed for assay, no further reactivation could occur in that aliquot, since it has been demonstrated that reoxidation of reduced RNase does not give an active product at pH 5.0 (the pH of the assay medium) (17).

Effect of Ribosomes—The acceleration of reactivation of reduced RNase was not observed when microsomes were replaced with rat liver ribosomes in the "standard reoxidation mixture." The possibility that the ribosome is the part of the microsome essential for activity is not ruled out by this experiment, however, since deoxycholate present in the ribosome preparation may have inhibited the reactivation process. Deoxycholate, in an amount roughly equal to that which had been added with the ribosomes, was able to reduce markedly the stimulatory effect of the "standard reoxidation mixture."

Ribosomes prepared from *Escherichia coli*[6] in the absence of deoxycholate (18) were also unable to replace liver microsomes in the "standard reoxidation mixture."

Effect of Replacement of S-II Fraction with Various Cofactors—Because of the experiments indicating that the active component(s) in the S-II fraction is of small size, an attempt was made to replace the S-II fraction with various small molecules of known biological importance. Among the compounds tested were DPN, DPNH, TPN, TPNH, GSSG, GSH, coenzyme Q_{10}, a mixture of 2′- and 3′-cytidylic acid, and various oligonucleotides of uridylic acid, cytidylic acid, and adenylic acid. None of the substances listed was active.

Effect of Various Substances on Complete System—EDTA, TPN, TPNH, GSSG, and GSH were added to reoxidation mixtures containing microsomes and the S-II fraction ("standard reoxidation mixtures"). None of these compounds stimulated the activity of the system. As shown in Table III, GSSG, at a concentration of 10^{-3} M, inhibited the system, but GSH was without effect. TPNH was able to reverse the inhibition completely, possibly by stimulating the conversion of the glutathione to its reduced form via a TPNH-glutathione reductase. The inhibitory effect of GSSG was also observed in the absence of liver fractions (pH 8.2, 24°).

Effect of pH—The kinetics of reactivation of reduced RNase in the "standard reoxidation mixture" were studied at pH 7.0, 7.4, 7.8, and 8.2. The results are shown in Fig. 4. In contrast to the marked pH dependence previously reported for the reactivation of reduced RNase in the absence of liver fractions (6), only small differences were noted in the present case, except that the rate of reactivation was moderately slower at pH 7.0 than at higher pH values.

Exposure of the microsomes to a pH between 5 and 6 for a few minutes at 24° resulted in clumping and irreversible loss of activity.

Effect of Temperature—The kinetics of reactivation of reduced RNase in the "standard reoxidation mixture" were studied at 24° and at 37°. Again, in contrast to the situation in the absence of liver fractions (6), no significant difference was observed.

Effect of Variations in Concentrations of Liver Fractions—A series of experiments was performed to determine the effect of variations in the concentrations of the microsomal and S-II fractions on the reactivation of reduced RNase. The results

[4] Kindly provided by Dr. William Carroll, National Institutes of Health, Bethesda, Maryland.

[5] Polyalaninated lysozyme was kindly prepared by Dr. Michael Sela, Weizmann Institute of Science, Rehovoth, Israel. This preparation, which was not enzymically active, was reduced by the same procedure as that used for native RNase.

[6] Kindly provided by Dr. Marshall Nirenberg, National Heart Institute, National Institutes of Health, Bethesda, Maryland.

are shown graphically in Fig. 5. In all cases the results have been corrected for the appropriate control values. In the presence of the standard quantity of the S-II fraction, a decrease in the amount of microsomes to one-tenth of the standard amount caused a large drop in activity. The activity of the system was unaffected, however, by a relatively large decrease in the amount of the S-II fraction, one-tenth of the standard amount being able to sustain most of the activity. It is of interest that diminution of the amount of the S-II fraction to one-third of the standard amount caused an increase in the activity of the reoxidation mixture (see "Discussion").

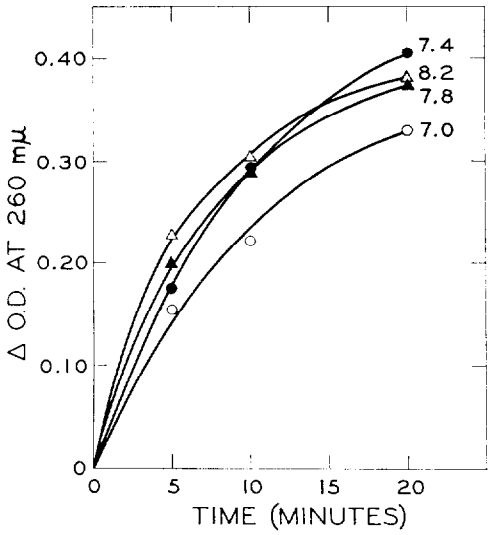

FIG. 4. Effect of pH on the rate of reactivation of reduced RNase. Reduced RNase, at a concentration of 0.018 mg per ml, was reoxidized in "standard reoxidation mixtures" at various pH values. Assays were performed immediately after the addition of reduced enzyme and again at various times over the following 20 minutes. The differences between the zero time activities and the 5-, 10-, and 20-minutes activities are expressed as the differences in optical density at 260 mμ in the assay tubes.

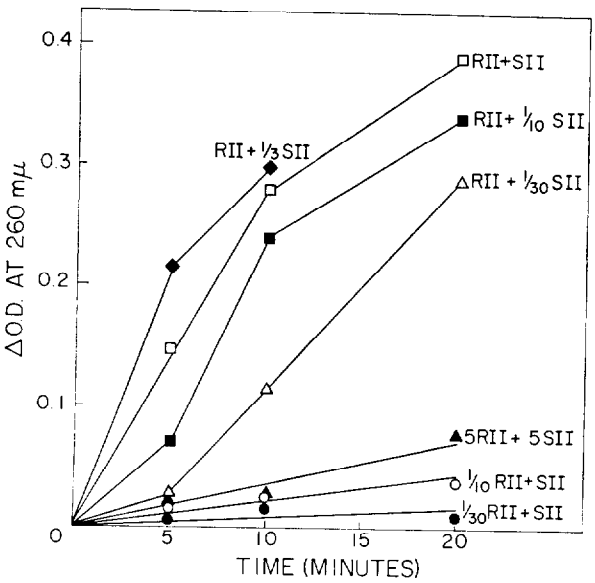

FIG. 5. Effect of variations in the concentrations of liver fractions on the rate of reactivation of reduced RNase. Reduced RNase, at a concentration of 0.018 mg per ml, was reoxidized in reoxidation mixtures containing various proportions of microsomes and the S-II fraction. The compositions of the mixtures are indicated as fractions or multiples of the standard amounts of microsomes and the S-II fraction. The differences between the activities measured immediately after the addition of reduced enzyme and those measured 5, 10, and 20 minutes later are expressed as the differences in optical density at 260 mμ in the assay tubes.

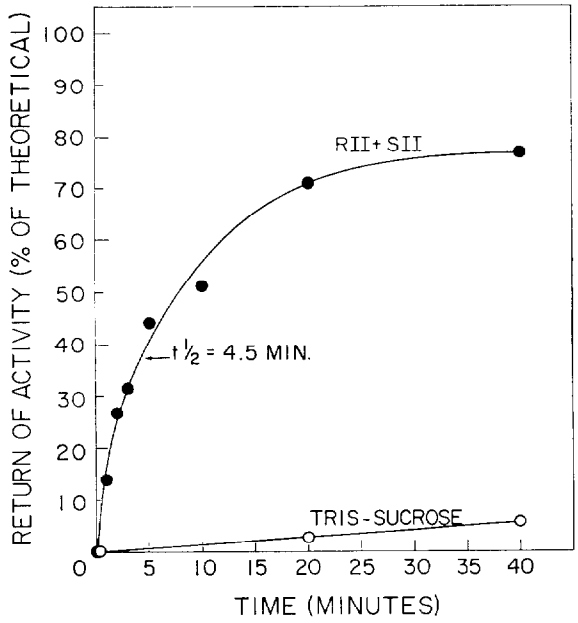

FIG. 6. Reactivation of reduced RNase at the maximal rate achieved. Reduced RNase, at a concentration of 0.018 mg per ml, was reoxidized in the presence of a reoxidation mixture containing 5 times the standard amount of microsomes and one-third the standard amount of the S-II fraction. Assays were performed immediately after the addition of reduced enzyme and again 1, 2, 3, 5, 10, 20, and 40 minutes later. The differences between the zero time activities and the other activities are plotted (*upper curve*) as percentages of the activity expected theoretically on the basis of complete conversion of the reduced RNase to native enzyme. At the same time, for comparison, the rate of reactivation of reduced RNase in 0.01 M Tris-chloride-0.25 M sucrose solution was also measured (*lower curve*). Except for the presence of liver fractions in the one case and their absence in the other, the conditions of the two experiments were the same.

On the basis of these observations, a reoxidation mixture was prepared with proportions of the components that would be expected to yield maximal acceleration of the reactivation of reduced RNase: 5 times the standard amount of microsomes and one-third the standard amount of the S-II fraction. Reactivation of the reduced enzyme in the presence of this mixture did, indeed, proceed more rapidly, as shown in Fig. 6. The process was essentially complete within 20 minutes, with a half-time of 4.5 minutes. For technical reasons, still higher concentrations of microsomes could not be tested.

Fractions prepared from rat liver in 0.88 M sucrose by the method of Siekevitz (10) gave the same results as those prepared in 0.25 M sucrose-0.01 M Tris-chloride.

DISCUSSION

The present study demonstrates the existence of a system in rat liver that is capable of greatly accelerating the conversion of reduced bovine pancreatic RNase to the enzymically active form. All of the stimulatory effect of whole liver homogenate

was obtained by recombination of washed microsomes (R-II fraction) with the supernatant fluid (S-II fraction), each of which was inactive alone. No requirement for mitochondria (R-I fraction) could be demonstrated. Although microsomes were equally active when prepared in 0.25 M sucrose and in 0.88 M sucrose, their activity was totally lost after exposure to pH 5 to 6 for a few minutes at 24°, and markedly diminished by exposure to deoxycholate at a concentration of 0.1%.

Fractionation of the S-II fraction revealed that the active component(s) is of small size (molecular weight not more than 1000 to 2000), is dialyzable, and is stable to heat (100° for 3 minutes) and acid (pH 2.0, 5 minutes, 24°). It appears that some of the active component(s) is bound to protein, and that this portion can be dissociated partially from the protein by acid treatment. It has been concluded that none of the protein in the S-II fraction is required for the stimulatory effect of the "standard reoxidation mixture," since both a protein-free fraction (B-2) and a fraction very low in protein (acid- and heat-treated S-II) are able to produce nearly the full stimulatory effect when combined with microsomes.

In a previous paper dealing with the reoxidation of reduced RNase in buffered solutions (6), the following points were noted. (*a*) The rate of reactivation is markedly dependent on pH, with the optimum between pH 8.0 and 8.5. (*b*) Both the rate and the extent of reactivation are markedly dependent on temperature. At 24°, a small amount of activity can be detected within 5 minutes, and full activity returns in 60 to 100 minutes, whereas at 37° no activity can be detected until after 30 minutes, and the maximal extent of reactivation is less than 40%. (*c*) Under optimal conditions (pH 8.2, 24°, protein concentration 0.01 mg per ml), the half-time for the reactivation process is approximately 20 minutes. In the present study, in which reactivation was accomplished in the presence of liver fractions, there was no diminution in the rate or the extent of reactivation at 37° as compared with 24°. Furthermore, large variations of pH (from 7.0 to 8.2) had relatively little effect on the reactivation process.

There have been several recent reports of systems for the enzymic reduction of protein disulfide bonds[7] (19, 20). The system isolated from yeast by Black and Blondel (19), which catalyzes the reduction of such compounds as oxidized glutathione, oxytocin, and insulin, requires two different enzymes in addition to TPNH. This system does not reduce RNase or bovine serum albumin. Both Tomizawa (20) and Katzen, Tietze, and Stetten[7] have described systems derived from liver that catalyze the reduction of the disulfide bonds of insulin. These systems require reduced glutathione and soluble enzymes. Katzen, Tietze, and Stetten have also mentioned the possibility that their system, with the use of GSSG, may catalyze the correct reformation of disulfide linkages. The system described in the present report differs from those mentioned above in that it does not appear to be a degradative one, intact microsomes are required (in addition to a factor or factors in the supernatant fraction), and such cofactors as the pyridine nucleotides and glutathione do not appear to be involved. Any enzyme that might be involved in the present system would have to be associated with the microsomes, since no protein material is required from the supernatant fraction. Furthermore, enzymes involved in the other systems mentioned were prepared by methods known to destroy the activity of the microsomes.

Experiments in which the quantities of the microsomal and supernatant fractions were varied demonstrate that the active component(s) of the supernatant fraction is present in excess in the "standard reoxidation mixture." A 10-fold decrease in the amount of the S-II fraction included in the reoxidation mixture caused only a small decrease in activity. A 3-fold decrease actually produced an increase in activity, presumably owing to a concomitant decrease in the amount of an RNase inhibitor that may be present in the supernatant fraction (21). This hypothesis is supported by the finding that an increase in the amount of the S-II fraction in the reoxidation mixture (employing a concentrate prepared by lyophilization) caused a marked decrease in activity. Furthermore, as shown in Table I, reactivation of reduced RNase in the presence of the S-II fraction alone proceeds more slowly than in buffered solutions containing no liver fractions.

In the presence of the standard amount of the S-II fraction, the activities of reoxidation mixtures were proportional to the quantity of microsomes that they contained. The fastest rate of reactivation of reduced RNase attained in the present study occurred with 5 times the standard amount of microsomes, and the half-time under these conditions was 4.5 minutes. This period of time approaches that estimated for the biosynthesis of lysozyme and the chains of hemoglobin, each of which contains polypeptide chains similar in length to that of RNase (2, 22). It must be kept in mind, moreover, that this rate was achieved in a system in which the concentration of microsomes is known to be rate-limiting.

At the present time, there is no evidence bearing on the relevance of this system to protein biosynthesis *in vivo*. In contrast to the "optimal" conditions for reactivation in buffered solutions, however, the conditions under which this system functions are not incompatible with physiological requirements. Furthermore, the requirement for microsomes in this system is of great interest in view of the known involvement of the microsome in the earlier stages of protein biosynthesis. It does not seem unreasonable to speculate that the folding of the polypeptide chain and formation of disulfide bonds occur *in vivo* at or near the site at which the chain is assembled. If this system does operate *in vivo*, it must be a relatively nonspecific one, since in the present study there was a difference in origin between the enzyme and the enzyme-reactivating system with respect to both species and organ. Thus, a mixture of rat liver microsomes and beef heart or rat liver supernatant was able to accelerate the reactivation of reduced bovine pancreatic RNase.[8]

SUMMARY

A system capable of accelerating the rate of reactivation of reduced bovine pancreatic ribonuclease has been isolated from rat liver. This system consists of microsomes and a soluble nonprotein factor or factors. In contrast to the marked temperature and pH dependence previously reported for the reactivation process in buffered solutions, reactivation by the micro-

[7] H. M. Katzen, F. Tietze, and DeW. Stetten, Jr., manuscript in preparation.

[8] Since submission of this paper we have received an unpublished manuscript from Drs. P. Venetianer and F. B. Straub, entitled "The Enzymatic Reactivation of Reduced Ribonuclease." In this report the authors describe a two-part system derived from pigeon and chicken pancreatic extracts that consists of a nondialyzable, heat-labile factor and a dialyzable, heat-stable factor.

somal system is relatively independent of temperature (between 24° and 37°) and pH (between 7.0 and 8.2). The nonprotein factor(s) in this system can be replaced by a beef heart supernatant fraction, but not by pyridine nucleotides, glutathione, or oligonucleotides of uridylic acid, cytidylic acid, and adenylic acid. The activity of the microsomal system is strongly dependent on the concentration of microsomes. By using the highest feasible concentration of microsomes, a reoxidation mixture was prepared that gave a half-time for the reactivation process of 4.5 minutes.

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